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Viability of ectomycorrhizal fungi following cryopreservation

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ABSTRACT

The use of ectomycorrhizal (ECM) fungi in biotechnological processes requires their maintenance over long periods under conditions that maintain their genetic, phenotypic, and physiological stability. Cryopreservation is considered as the most reliable method for long-term storage of most filamentous fungi. However, this technique is not widespread for ECM fungi since many do not survive or exhibit poor recovery after freezing. The aim of this study was to develop an efficient cryopreservation protocol for the long-term storage of ECM fungi. Two cryopreservation protocols were compared. The first protocol was the conventional straw protocol (SP). The mycelium of the ECM isolates was grown in Petri dishes on agar and subsequently collected by punching the mycelium into a sterile straw before cryopreservation. In the second protocol, the cryovial protocol (CP), the mycelium of the ECM isolates was grown directly in cryovials filled with agar and subsequently cryopreserved. The same cryoprotectant solution, freezing, and thawing process, and re-growth conditions were used in both protocols. The survival (positive when at least 60 % of the replicates showed re-growth) was evaluated before and immediately after freezing as well as after 1 week, 1 m, and 6 m of storage at −130 °C. Greater survival rate (80 % for the CP as compared to 25 % for the SP) and faster re-growth (within 10 d for the CP compared to the 4 weeks for the SP) were observed for most isolates with the CP suggesting that the preparation of the cultures prior to freezing had a significant impact on the isolates survival. The suitability of the CP for cryopreservation of ECM fungi was further confirmed on a set of 98 ECM isolates and displayed a survival rate of 88 % of the isolates. Only some isolates belonging to *Suillus luteus*, *Hebeloma crustuliniforme*, *Paxillus involutus* and *Thelephora terrestris* failed to survive. This suggested that the CP is an adequate method for the ultra-low cryopreservation of a large set of ECM fungi and that further studies are necessary for the more recalcitrant ones.

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Introduction

Ectomycorrhizal (ECM) fungi are key organisms in soil–plant interactions in forestry ecosystems (Smith & Read 2008). Their application in timber production, afforestation, and bioremediation, and for some, their production as edible mushrooms, is increasing continuously (Fazenda *et al.* 2008; Duponnois *et al.* 2011; Karwa *et al.* 2011; Siddiqui & Kataoka 2011; Repáč 2011). However, the utilization of ECM fungi requires the selection and isolation of strains of high biotechnological and economical value and their preservation over long periods under conditions that maintain their genetic, phenotypic, and physiological stability (Kuek 1994; Ryan *et al.* 2001; Ryan & Smith 2004; Stacey & Day 2007; Duponnois *et al.* 2011; Repáč 2011).

The most common method to preserve ECM fungi in culture collections is continuous subculture on agar slants (Kuek 1994; Corbery & Le Tacon 1997; Siddiqui & Kataoka 2011). However, this method is prone to contaminations by other microorganisms or mites and is labour-intensive and space-consuming (Smith & Onions 1994). Moreover, the long-term genetic and phenotypic stability of the strains cannot be guaranteed as continuous subculturing can lead to mutations and selection pressure on the organism. A decrease in the ability of fungal isolates to form ECM associations after repeated sub-cultivations has been reported in several studies and has been associated with a reduced effectiveness of the fungi to improve plant growth (Marx & Daniel 1976; Thomson *et al.* 1993).

Cryopreservation at ultra-low temperature is considered as the most reliable method for long-term storage of most filamentous fungi (Smith 1998). Cryogenic storage is presumed to allow very long periods of storage (i.e. centuries or even millennia) and to prevent genetic and phenotypic changes (Mazur 1984). Moreover, it obviates the need for labour-intensive serial subcultures, requires few spaces. Cryopreservation of filamentous fungi was initiated about half a century ago by Hwang (1960) and has since then been widely and successfully applied to a wide range of fungi from most taxonomic groups and ways of life. However, some fungi are still difficult to cryopreserve. Problematic organisms include obligate symbionts or parasites (Ryan & Smith 2004) and some members of the *Basidiomycota* (Ryan & Smith 2004; Tan & van Ingen 2004). For the first group, Declerck & Angelo-Van Coppenolle (2000) reported the successful cryopreservation of *Rhizophagus* sp. (formerly *Glomus intraradices*) MUCL 41835, an arbuscular mycorrhizal fungus, while Ryan & Ellison (2003) succeeded in the ultra-low preservation of *Puccinia spegazzinii*, a microcyclic rust-fungus.

Among the *Basidiomycota*, the lowest survivals following cryopreservation were recorded for ECM fungi (Tan & van Ingen 2004). Many of them do not survive or exhibit poor recovery after freezing (Ito & Nakagiri 1996; Corbery & Le Tacon 1997; Smith 1998; Yang & Rossignol 1998; Danell & Flygh 2002; Obase *et al.* 2011). The cryopreservation methods are mainly based on agar plugs as carriers of the fungal mycelium placed into ampoules, straws, and/or cryovials (Ryan & Smith 2004; Day *et al.* 2008). Cryopreservation is undertaken using a controlled cooling rate (mostly of $-1\ ^{\circ}C\ min^{-1}$) coupled with a cryoprotectant such as glycerol.

ECM fungi present several features that make the implementation of cryopreservation protocols rather delicate. These organisms do not produce conidia (Hutchison 1989) and the sexual structures are rarely formed on synthetic media. Their most common form *in vitro* is mycelium that generally grows slowly in axenic culture (Heinonen-Tanski 1990; Molina *et al.* 2002; Homolka *et al.* 2003). The vegetative hyphae are more sensitive to freezing than fungal spores (Tan *et al.* 1991; Smith 1993). Indeed, as the temperature decreases, ice crystals form preferentially where the hyphal wall has been damaged, for instance during the sample preparation (Tan & van Ingen 2004). Once formed, the ice propagates through the hyphal cell and the septa until all the interconnected hyphae are crystallized (Coulson *et al.* 1986; Smith 1993; Smith & Thomas 1998; Tan & van Ingen 2004).

In the last decade, alternative methods for cryopreservation emerged for several recalcitrant basidiomycetes such as edible mushrooms, wood-decaying or saprotrophic fungi. Most of these techniques were based on the use of various carrier materials for the mycelial growth such as sawdust (Kitamoto *et al.* 2002), perlite (Homolka *et al.* 2001, 2006, 2007, 2010) or cereal grains (Mata *et al.* 2000, 2003; Colauto *et al.* 2011). Recently a technique based on charcoal filter paper was proposed for the cryopreservation of some ECM and saprotrophic basidiomycetes and ascomycetes (Stielow *et al.* 2012). This two-steps technique consisted in growing the fungal strains on charcoal filter paper strips placed on a synthetic solid medium. The overgrown strips are then removed and introduced in cryovials for the freezing process. Four out of eight ECM species (i.e. six strains out of ten) were successfully maintained at $-196\ ^{\circ}C$ with this technique.

In the present study, we present another efficient and simple method for the long-term storage of ECM fungi. This single-step technique, adapted from Voyron *et al.* (2009), is based on the direct growth of the ECM fungi in cryovials prior to storage at ultra-low temperature. The viability of ECM fungal isolates belonging to different genera and species was, in a first step, assessed after cryopreservation and compared to the viability using the conventional straw technique (Hoffmann 1991), one of the most widely used techniques for the cryopreservation of filamentous fungi and slightly modified in our study. The cryopreservation method was evaluated, in a second step, on a set of 98 ECM isolates to confirm its suitability for the cryopreservation of numerous ECM fungi.

Materials and methods

ECM fungi

A total of 113 isolates belonging to ten ECM fungal genera and 12 species were considered (Table 1). The viability of 15 isolates was first assessed using two cryopreservation protocols. Ninety-eight additional isolates were then considered for the validation part of the experiment. Since their isolation, the fungal isolates were maintained in culture collection by regular transfer on the modified Fries Medium (MFM; Colpaert *et al.* 2000) at pH 4.8 and incubated at 4 °C in the dark.

Table 1 – ECM fungal isolates considered for cryopreservation at −130 °C.

Genus and species	Isolate[a]	Collecting date	% viability[e]
Development of a cryopreservation protocol			
Hebeloma crustuliniforme	52208 (Hc1)	1998	–
Hymenoscyphus sp.	52205 (ARON 2893/S)[b]	1998	–
Laccaria bicolor	52210 (LbicLs1)	2005	–
Paxillus involutus	52215 (Pi2)	1995	–
	52217 (Pi23)	1998	–
Pisolithus tinctorius	52222 (Pt14) [c]	Unknown	–
Rhizopogon luteolus	52199 (E1Rlu)	2000	–
	52200 (P1Rlu)	2000	–
	52202 (Ls2Rlu)	2000	–
Suillus bovinus	52173 (P2Sbo)	2000	–
	52176 (P5Sbo)	2000	–
Suillus luteus	52104 (MG1Slu)	2000	–
	52105 (MG2Slu)	2000	–
	52135 (HH18Slu)	2000	–
Thelephora terrestris	52223 (Tt)	1998	–
Validation of the CP			
Cortinarius sp.	52207 (Cosp51)	1998	100 %
Hebeloma crustuliniforme	52209 (Hc50)	1998	0 %
Laccaria bicolor	52211 (LbicLs2)	2005	100 %
	52212 (LbicLs5sp)	2005	100 %
	52213 (LbicLs6sp)	2005	100 %
Lactarius rufus	52214 (Lr30)	1998	100 %
Paxillus involutus	52218 (Pi30)	1998	100 %
	52219 (Pi50)	1998	0 %
	52220 (Pi51)	1998	80 %
Suillus bovinus	52149 (N1Sbo)	2000	100 %
	52150 (N2Sbo)	2000	100 %
	52151 (N3Sbo)	2000	100 %
	52152 (N5Sbo)	2001	100 %
	52153 (N7Sbo)	2001	100 %
	52154 (E1Sbo)	2000	100 %
	52155 (E2Sbo)	2000	100 %
	52156 (E3Sbo)	2001	100 %
	52157 (E4Sbo)	2001	100 %
	52158 (MG3Sbo)	2001	100 %
	52159 (MG4Sbo)	2001	100 %
	52160 (MG5Sbo)	2001	100 %
	52161 (MG6Sbo)	2001	100 %
	52162 (Ls1Sbo)	2000	100 %
	52163 (LS2Sbo)	2000	100 %
	52164 (Ls3Sbo)	2000	100 %
	52165 (Ls4Sbo)	2000	100 %
	52166 (Lst1Sbo)	2000	100 %
	52167 (Lst4Sbo)	2000	100 %
	52168 (Lst6Sbo)	2000	100 %
	52169 (Lst8Sbo)	2000	100 %
	52170 (HR1Sbo)	2000	80 %
	52171 (HH1Sbo)	2001	100 %
	52172 (P1Sbo)	2000	100 %
	52174 (P3Sbo)	2000	100 %
	52175 (P4Sbo)	2000	100 %
	52177 (OF1Sbo)	2001	80 %
	52178 (OF2Sbo)	2001	100 %
	52179 (OF3Sbo)	2001	100 %
	52180 (OF4Sbo)	2001	100 %
	52181 (Z1Sbo)	2001	100 %
	52182 (Z2Sbo)	2001	100 %
	52183 (Z3Sbo)	2001	100 %
	52184 (Z4Sbo)	2001	100 %
	52185 (DS1Sbo)	2003	100 %
	52186 (DS2Sbo)	2003	100 %
	52188 (DS4Sbo)	2003	100 %

(*continued on next page*)

Table 1 – (continued)

Genus and species	Isolate[a]	Collecting date	% viability[e]
Suillus luteus	52100 (E1Slu)	2000	100 %
	52101 (E2Slu)	2000	100 %
	52102 (Ew2Slu)	2001	100 %
	52103 (Ew3Slu)	2001	60 %
	52106 (MG4Slu)	2000	60 %
	52107 (MG5Slu)	2000	60 %
	52108 (N1Slu)	2000	80 %
	52109 (N2Slu)	2000	40 %
	52110 (N3Slu)	2000	50 %
	52111 (Na2Slu)	2000	75 %
	52112 (Na3Slu)	2000	60 %
	52113 (Na4Slu)	2000	100 %
	52114 (A9Slu)	2001	100 %
	52115 (A14Slu)	2001	40 %
	52116 (MM1Slu)	2002	100 %
	52117 (P1Slu)	2000	0 %
	52118 (P2Slu)	2000	100 %
	52119 (P4Slu)	2000	60 %
	52120 (P8Slu)	2000	80 %
	52121 (LM2Slu)	2000	100 %
	52122 (LM3Slu)	2000	80 %
	52123 (LM4Slu)	2000	100 %
	52124 (Ls1Slu)	2000	100 %
	52125 (Ls2Slu)	2000	80 %
	52126 (Ls3Slu)	2000	100 %
	52127 (Lst1Slu)	2000	100 %
	52128 (Lst2Slu)	2000	75 %
	52129 (Lss4Slu)	2000	100 %
	52130 (Lss5Slu)	2000	60 %
	52131 (Lss6Slu)	2000	100 %
	52132 (Lss44Slu)	2000	80 %
	52133 (HH1Slu)	2000	80 %
	52134 (HH3Slu)	2000	100 %
	52136 (HH19Slu)	2000	75 %
	52137 (HR1Slu)	2000	0 %
	52138 (HR6Slu)	2000	25 %
	52139 (HR7Slu)	2000	100 %
	52140 (HR8Slu)	2000	60 %
	52141 (OF1Slu)	2000	25 %
	52142 (OF2Slu)	2000	80 %
	52143 (OF3Slu)	2000	20 %
	52144 (OF5Slu)	2000	100 %
	52145 (DS2Slu)	2003	0 %
	52146 (DS3Slu)	2003	100 %
	52147 (DS4Slu)	2003	80 %
	52148 (DS5Slu)	2003	100 %
Suillus variegatus	52189 (P1Sva)	2001	100 %
	52190 (P2Sva)	2001	100 %
	52191 (Z1Sva)	2001	100 %
	52192 (Z3Sva)	2001	100 %
	52193 (T3.1Sva)[d]	2001	100 %
	52194 (T4.1Sva)[d]	2001	100 %

The % viability of the isolate was considered following two cryopreservation methods: the SP and the CP were evaluated before and immediately after freezing as well as after 1 week, 1 m, and 6 m of storage at −130 °C.

a The first number refers to the code number assigned to the isolate in the Mycothèque de l'Université catholique de Louvain (MUCL), Louvain-la-Neuve, Belgium and the number between brackets refers to the origin code of the isolate. The isolates were collected by Prof. Colpaert in the province of Limburg (Belgium) from *Pinus* sp. except.

b Collected by Vrålstad in Telemark (Norway) from *Quercus robur*.

c Collector, locality, and plant host are unknown.

d Collected by Vrålstad in Hedmark (Norway) from *Pinus* sp.

e Percentage of mycelial plugs that re-grew after cryopreservation.

Cryopreservation protocols and fungal cultures preparation
Two protocols differing in the mode of preparation of the cultures were tested and compared.

The first protocol, the straw protocol (SP), was adapted from the protocol of Hoffmann (1991). The ECM fungal isolates were grown in Petri dishes (92 mm diameter) on sterilized (121 °C for 15 min) MFM (approximately 30 ml) solidified with 13.5 g l^{-1} of agar (Scharlau, Spain). The cultures were incubated in the dark at 22–23 °C for 2–4 weeks depending on the growth of each isolate. Actively growing colonies were then flooded with 10 ml of a sterilised (121 °C for 15 min) glycerol solution (10 % v/v) and subsequently incubated for 1–2 h to allow the cryoprotectant to penetrate the cells before freezing (Hubálek 2003). Sterile polypropylene straws (Laboratoire Humeau, France) of 4 mm in diameter and 37 mm in length, open at both ends were used to sample the mycelium and the underlying agar at the margin of the colony. Five mycelium plugs were punched into each straw that was transferred into a 2 ml sterile polypropylene microtube (Sarstedt, Germany) used as cryovial.

The second protocol, the cryovial protocol (CP), was adapted from the protocol of Voyron *et al.* (2009) developed for the cryopreservation of white-rot fungi. The 15 ECM fungal isolates were grown on approximately 30 ml MFM agar in Petri dishes (92 mm diameter) and incubated in the dark at 22–23 °C for 2–4 weeks. A small mycelium plug (approximately 4 mm × 4 mm) was sampled at the margin of the growing colonies and inoculated into a 2 ml sterile polypropylene microtube (Sarstedt, Germany) used as cryovial containing 750 µl of sterilized (121 °C for 15 min) MFM agar (10 g l^{-1}) poured in a slope. Cryovials were incubated at 22–23 °C in the dark for 7–9 weeks. A 500 µl sterilized (121 °C for 15 min) glycerol solution (10 % v/v) was added into the cryovial 1–2 h before cryopreservation.

Freezing rates
The cultures were progressively cooled in a controlled rate freezer (Icecube 1600, SY-Lab, Austria) as follows: 8 °C min^{-1} from +20 °C to +4 °C; 1 °C min^{-1} from +4 °C to −50 °C; 10 °C min^{-1} from −50 °C to −100 °C. The cultures were then directly transferred into a freezer (SANYO, Japan) at −130 °C.

Cryopreservation treatments
Three storage durations at −130 °C were tested: 1 week, 1 m, and 6 m. Control cultures (i.e. without freezing) were included to check that the addition of glycerol had no detrimental effect on the viability of the cultures. Furthermore, a set of isolates was immediately thawed after freezing to test the effect of the early freezing process (i.e. 0 h incubation). In total five treatments (i.e. early freezing (0 h incubation), 1 week, 1 m, and 6 m cryopreservation and non-cryopreserved control) were considered with ten cryovials containing the straws for the SP and 15 cryovials for the CP, (except for the Pt14 control cultures that counted only ten replicates).

Thawing and revival of the cultures
For both methods, cryovials were immersed directly into a water bath at 38 °C for about 2 min. The agar plugs were then transferred in Petri dishes (92 mm diameter) on approximately 30 ml MFM and incubated at 22–23 °C for 4 weeks. For the SP, the five plugs originating from a single straw were transferred in a single Petri dish; whereas for the CP, the whole colony was carefully transferred onto the centre of the Petri dish.

Viability assessments
For the SP, a survival rate of at least 60 % of the plugs in a single Petri plate after 4 weeks of incubation was coded as one; while a survival rate inferior to 60 % was coded as zero. Means in percent were then calculated for the ten replicates per treatment. For the CP, the viability was estimated by the percentage of re-growth of the mycelium from each plug. Because ECM fungi are known to exhibit usually poor recovery after cryopreservation, a viability rate of at least 60 % was considered as an acceptable result for the long-term storage of those organisms.

Statistical analysis
In order to test whether the protocol had an effect on viability, the data after freezing were analyzed with a general linear mixed model (GLMM) with binary error distribution and logit-link function. The cryopreservation protocols and the cryopreservation conditions were analyzed as fixed effects. The ECM fungal isolates were used as a random effect. The analyses were performed using a GLIMMIX procedure in the Statistical Analysis System (SAS) software (SAS 9.3). The same model was examined separately for SP and CP to analyze the effect of the cryopreservation treatment on viability. In order to run comparisons between the cryopreservation treatments after freezing the LSMESTIMATE statement was used with Bonferroni correction for multiple comparisons (experiment wise alpha = 0.05).

Validation of the CP
The CP described above was applied except that the cultures inoculated in cryovials onto solid MFM were incubated for 4 weeks instead of 7–9 weeks, because no difference in viability was observed in additional tests on three isolates (MUCL 52208, 52210, and 52217) (data not shown). Five replicates were inoculated per isolate. All isolates were tested for viability (survival rate of at least 60 %) after 1 m of preservation at −130 °C.

Results

Viability of ECM fungi following two protocols of cryopreservation

Three isolates, *Paxillus involutus* MUCL 52215, MUCL 52217, and *Pisolithus tinctorius* MUCL 52222 could not be evaluated with the SP since they failed to survive in preliminary tests using this technique (data not shown).

Whatever the protocol tested, the controls showed high viability rates (Table 1). With the exception of *P. tinctorius* MUCL 52222 (90 % viability), the viability rate of the ECM fungal isolates was 100 %.

For the SP at early freezing (0 h incubation), a survival rate above 60 % was observed for six (MUCL 52205, 52202, 52173, 52176, 52135, and 52223) out of the 12 isolates. Five isolates stored 1 week at −130 °C (MUCL 52205, 52202, 52176, 52135, and 52223) or 1 m (MUCL 52205, 52173, 52176, 52135, and

52223) at −130 °C exhibited a survival rate above the 60 % survival threshold. After 6 m of storage at −130 °C, only three isolates (MUCL 52205, 52176, and 52223) presented a survival rate above 60 %.

For the CP, the 15 isolates had a recovery rate above 60 % at the early freezing (0 h incubation). Eleven isolates (MUCL 52208, 52205, 52210, 52215, 52217, 52199, 52200, 52202, 52173, 52176, and 52135) exhibited a recovery rate above 60 % after 1 week. After 1 m and 6 m of storage at −130 °C, 12 isolates (MUCL 52208, 52205, 52210, 52215, 52217, 52222, 52199, 52200, 52202, 52173, 52176, and 52135) presented a survival rate equal or above the 60 % threshold.

The viability after freezing significantly (*P*-value <0.0001) differed between the two-cryopreservation protocols. With the exception of *Hymenoscyphus* sp. MUCL 52205 (at 0 h, 1 week, 1 m, and 6 m), *Suillus bovinus* MUCL 52176 (at 0 h and 1 m), *Suillus luteus* MUCL 52135 (at 0 h and 1 week) and *Thelephora terrestris* MUCL 52223 (at 0 h, 1 week, 1 m, and 6 m), the CP allowed a higher survival rate than the SP for the ECM tested (Fig 1).

A highly significant effect (*P*-values < 0.0001) of the treatment on the viability of the ECM fungal isolates was observed for both protocols. For the SP, the viability did not decrease significantly from 0 h to 1 week (*P*-value = 0.1062) and from 1 week to 1 m of storage (*P*-value = 1) while a highly significant decrease was observed from 1 m to 6 m (*P*-value = 0.0009). For the CP, a highly significantly decrease was observed from 0 h to 1 week (*P*-value <0.0001), although the viability rates remained above the 60 % threshold for 11 out of 15 isolates (Fig 1). After 1 week, no significant differences in viability with increasing time of storage were observed (1 week-1 m: *P*-value = 0.5115; 1 m−6 m: *P*-value = 0.4033).

The incubation period required to re-initiate growth following cryopreservation was reduced with the CP as compared to the SP. Indeed, after 6 m of storage at −130 °C, most isolates had initiate re-growth in a period of 10 d with the CP, while a period of 4 weeks was necessary with the SP (data not presented).

Validation of the CP

On the 98 isolates tested, 87 isolates exhibited a survival rate equal or above to the 60 % threshold (i.e. 89 % of the isolates) and 66 isolates exhibited 100 % of viability after 1 m of storage (i.e. 67 % of the isolates) (Tables 1 and 2). Among the 11 isolates that exhibited a survival rate below the 60 % threshold, nine

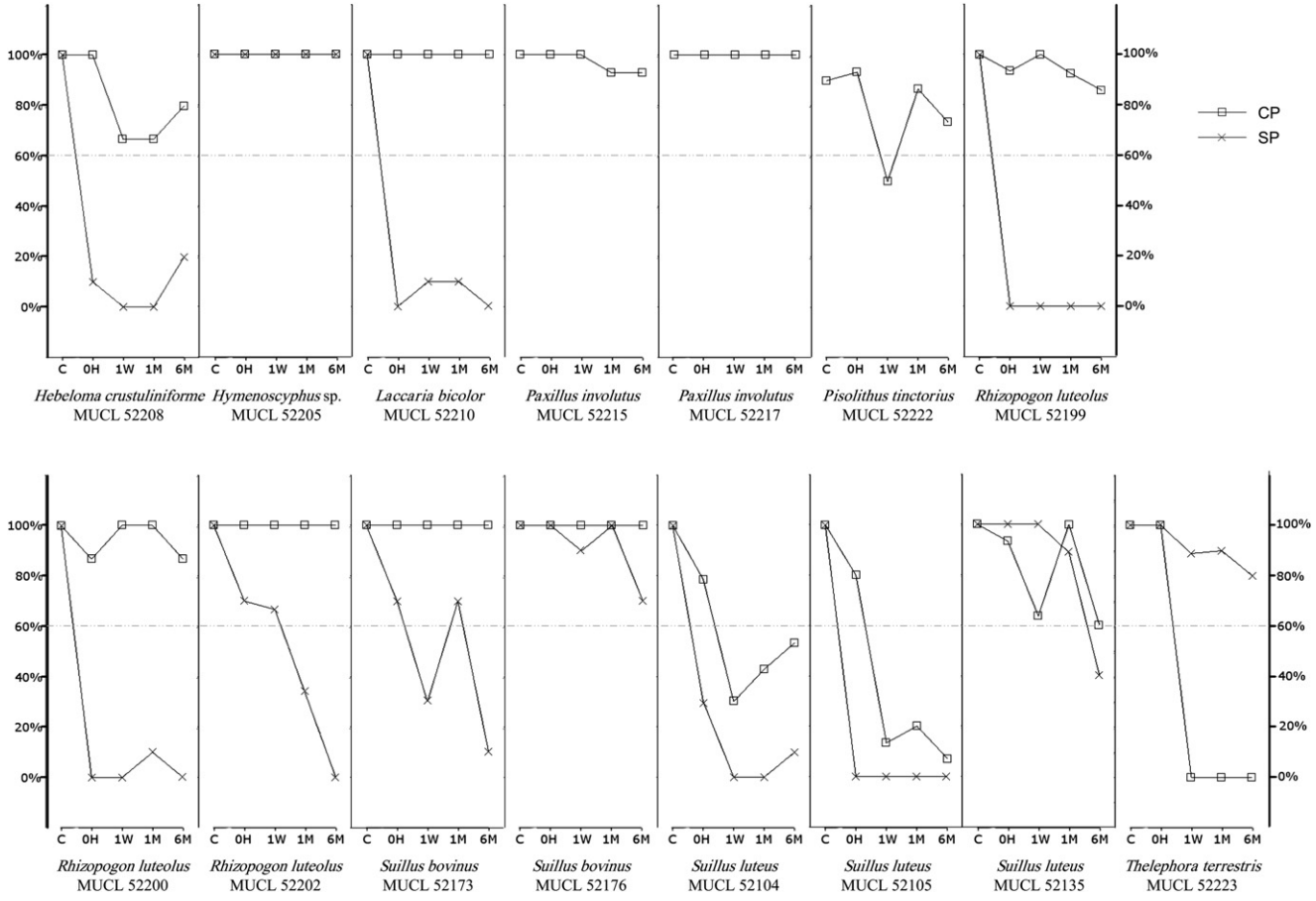

Fig 1 − Percentages of viability of the 15 ECM fungal isolates following two cryopreservation methods: (1) the SP (represented by the crosses) and (2) the CP (represented by the squares). The different cryopreservation conditions are symbolized by C for the controls (i.e. non-cryopreservation); 0H (immediate thawing after freezing); 1 W, 1 M, and 6 M (cryopreservation for 1 week, 1 m, and 6 m before thawing). The dotted line represents the 60 % of viability threshold considered as an acceptable result for the long-term maintenance of ECM fungal cultures. Three isolates (MUCL 52215, 52217, and 52222) were not tested with the SP since they failed to survive in preliminary attempts.

Table 2 – Viability of ECM isolates after 1 m storage at −130 °C using the CP.

Genus and species	Nb of tested isolates	Nb of isolates exhibiting a viability rate equal or above the 60 % threshold	Nb of isolates exhibiting 100 % in viability
Cortinarius sp.	1	1	1
Hebeloma crustuliniforme	1	0	0
Laccaria bicolor	3	3	3
Lactarius rufus	1	1	1
Paxillus involutus	3	2	1
Suillus bovinus	37	37	35
Suillus luteus	46	37	19
Suillus variegatus	6	6	6
Total	98	87	66

out of the 46 (i.e. 20 %) belonged to *Suillus luteus*. In addition, the *Hebeloma crustuliniforme* isolate and one *Paxillus involutus* isolate exhibited no recovery.

Discussion

The aim of this study was to develop a long-term preservation method at ultra-low temperature for ECM fungi. The viability of ECM fungal isolates belonging to different genera and species was assessed using two cryopreservation protocols differing in the mode of preparation of the cultures: the CP and the SP. The cryoprotectant solution, the freezing, and thawing process, the durations of storage and the revival conditions were identical in both protocols. The CP resulted in a greater survival rate of the ECM isolates (80 %) as compared to the SP (25 %), suggesting that the preparation of the culture is a key factor for the survival of ECM fungi at ultra-low temperature.

Among the cryoprotectants used in culture collections for filamentous fungi, the most common is glycerol (Hubálek 2003). It is reported to have low toxicity, even if damage to cells has already been reported after prolonged incubation (Ryan & Smith 2007). In our experiment, no toxic effect on the viability of the ECM strains was detected before cryopreservation.

The major advantage of the CP over the SP is that the cultures were grown directly in the cryovials, avoiding damages to cultures caused by handling (i.e. transfer from culture to straw). Indeed, mechanical damages to the mycelium may predispose the organisms to further injuries during the freezing process (Smith & Onions 1994) since ice crystals form preferentially where the hyphal wall has been damaged (Tan & van Ingen 2004). This probable reduction of the freezing damages may further explain the rapid re-growth following thawing of nearly all the isolates tested. This hypothesis was also reported by Homolka *et al.* (2001, 2006). These authors observed that some basidiomycetes, that generally failed to survive routine cryopreservation protocols, had high survival rates when grown directly in cryovials on perlite. Stielow *et al.* (2012), using charcoal filter papers as carrier of cultures before cryopreservation of some ECM and saprotrophic basidiomycetes and ascomycetes formulated the same hypothesis. The fungi grown on the filter paper were not damaged before transfer in the cryovials.

The second advantage of the CP over the SP is that cryovials in the CP contained a higher density of hyphae as compared to the SP. Indeed, whereas the straws were 4 mm in diameter, the cryovials were 10 mm in diameter. Moreover, the synthetic medium was poured on slants into the cryovials that increases the agar surface for fungal growth (i.e. approximately 12.5 mm^2 in the straw *vs.* approximately 150 mm^2 in the cryovial). Increasing the density of hyphae may increase the probability that some hyphae have survived cold stress and therefore may initiate re-growth more promptly and faster.

Finally, the physiological state of the cultures at the initiation of cryopreservation may have influenced the survival to cryogenic storage, as reported by several authors (Morris *et al.* 1988; Smith 1993; Smith 1998; Tanghe *et al.* 2003). The ECM fungal cultures in the CP were older (7–9 weeks in the first test) than in the SP (2–4 weeks). Only the youngest hyphae at the margin of the colony were sampled in the SP, whereas the whole colony was frozen in the CP. Moreover, because of the relatively small size of the cryovials compared to the Petri dishes, the growth of the cultures was more limited for the CP than for the SP. Consequently, cultures were probably in a later physiological stage of their growth in the CP than the SP even after 4 weeks of incubation as in the validation phase of the CP. This is consistent with the general statement that filamentous fungi residing in the late exponential phase and stationary phase survive freezing better than actively growing cultures (Tanghe *et al.* 2003; Tan & van Ingen 2004), even though most successful cryopreservation protocols of basidiomycetes used actively growing cultures (e.g. Chvostová *et al.* 1995; Yang & Rossignol 1998; Croan *et al.* 1999; Homolka *et al.* 2003; Voyron *et al.* 2009).

Besides the cultures ages, the growth conditions differed also in both protocols and may have influenced the survival to freezing. The releases of auto-inhibitory metabolites, staling substances, and the depletion of nutrients in the culture medium were probably more stringent in the cryovials for the CP than in the Petri dishes for the SP even after 4 weeks of growth as in the validation phase of the CP. In addition, the cryovials were screwed down during the growth of the cultures, resulting probably in a reduction of the oxygen level. Under these conditions, the cultures inside the cryovials might have switched to a stage where the metabolism is slowed-down before the cryopreservation process took place and triggered mechanisms to adapt to the stress conditions. The exposure of yeasts and filamentous fungi under stressful conditions before cryopreservation was shown to result in an increase in viability as several mechanisms involved in freeze tolerance are common with the protecting responses to other stresses (osmotic, oxidative, temperature stresses, …) (Smith & Onions 1994; Tanghe *et al.* 2003; Tan & van Ingen 2004). Several authors (Smith 1993; Smith & Onions 1994; Ryan & Smith 2007) reported on the contrary that cultures grown under optimal growth conditions rather than cultures grown under stressful conditions exhibit the highest viability. The reduction of the period of incubation in the validation phase might actually have positively affected the viability of *Suillus luteus* isolates after cryopreservation since in the first phase of the

experiment only one out three *S. luteus* isolates (i.e. 33 %) exhibited survival rate above 60 % *vs.* 37 out 46 isolates (i.e. 80 %) in the validation phase. Accordingly, to improve the CP for the few recalcitrant ECM isolates belonging to *S. luteus*, *Hebeloma crustuliniforme*, *Paxillus involutus*, and *Thelephora terrestris* (see Tables 1 and 2), additional studies should be conducted to evaluate the effects of the growth conditions and the age of the cultures on the physiological state of these fungi to understand the factors which are primordial for their successful ultra-low preservation.

An important decrease in viability was observed for some samples from the 0 h condition and the 1 week condition with the CP. Because the two treatments differed in both the temperature (−100 °C *vs.* −130 °C) and the duration of storage (i.e. immediately thawed *vs.* stored 1 week), it is not possible to determine if the loss of viability could be attributed to the decrease in temperature or to the duration of storage. Nevertheless, no further reduction of viability was observed between 1 week and 6 m of storage for the CP whereas a significant decrease was observed using the SP. The stability of the viability of the ECM fungal isolates with increasing time of storage observed with the CP supported the higher reliability of that protocol as compared to conventional cryopreservation methods (such as the straw technique) for the long-term storage of ECM fungi.

In addition to the higher survival and faster re-growth following thawing, the CP presented several technical advantages. The growth of the culture inside the cryovials can be checked before freezing in order to discard the poorly developed or non-viable cultures. The cryovial technique requires less manipulation than most of the other techniques and consequently the risk of contaminations is reduced.

Finally, the successful application of the protocol for 89 % of the isolates (i.e. 87 out of 98 isolates exhibited survival rates above 60 %) suggested its adaptability to a large set of ECM fungi.

In conclusion, the CP is a promising suitable and easy-to-apply procedure for the maintenance of a large set of ECM fungi. It requires some further adjustments for few ECM isolates (belonging to *S. luteus, H. crustuliniforme*, *P. involutus*, and *T. terrestris*). For those isolates, the influence of some parameters (e.g. culture age, oxygen stress…) on the physiological state of the organisms and their subsequent survival to freezing should be investigated in order to preserve them at their optimal stage of development. Additional experiments are conducted to ensure that ECM isolates maintained their genetic, phenotypic, and physiological traits after long-term cryopreservation.

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